REVIEW

Efflux-mediated multiresistance in Gram-negative bacteria

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ABSTRACT

Multiresistance in Gram-negative pathogens, particularly *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Acinetobacter* spp. and the Enterobacteriaceae, is a significant problem in medicine today. While multiple mechanisms often contribute to multiresistance, a broadly distributed family of three-component multidrug efflux systems is an increasingly recognised determinant of both intrinsic and acquired multiresistance in these organisms. Homologues of these efflux systems are also readily identifiable in the genome sequences of a wide range of Gram-negative organisms, pathogens and non-pathogens alike, where they probably promote efflux-mediated resistance to multiple antimicrobials. Significantly, these systems often accommodate biocides, raising the spectre of biocide-mediated selection of multiresistance in Gram-negative pathogens. While there is some debate as to the natural function of these efflux systems, only some of which are inducible by their antimicrobial substrates, their contribution to resistance in a variety of pathogens nonetheless makes them reasonable targets for therapeutic intervention. Indeed, given the incredible chemical diversity of substrates accommodated by these efflux systems, it is likely that many novel or yet to be discovered antimicrobials will themselves be efflux substrates and, as such, efflux inhibitors may become an important component of Gram-negative antimicrobial therapy.

Keywords Efflux, gram-negative, multidrug, multiresistance, resistance

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INTRODUCTION

While it is clear that bacterial resistance to antimicrobials pre-dates their clinical use, medical and agricultural practices of the past 50 years or more have promoted resistance development and spread in both human and animal pathogens, thereby compromising effective chemotherapy of infectious diseases. While much attention is focused currently on Gram-positive and mycobacterial pathogens, Gram-negative bacteria, particularly those exhibiting a multiresistance phenotype, can still pose a serious infectious disease threat [1,2]. Resistance to multiple antibiotics is increasingly seen in the Enterobacteriaceae, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Acinetobacter* spp. [3]. Significantly, infections caused by such multiresistant Gram-negative pathogens are associated with excess morbidity and mortality [4–8].

Resistance to antibiotics occurs typically as a result of drug inactivation or modification, target alteration, or reduced accumulation associated with decreased permeability and/or increased efflux [9]. It may be an intrinsic feature of an organism, or may result from mutation or the acquisition of exogenous resistance genes [10,11]. Thus, multiresistance typically results from the accumulation of multiple mutations and/or resistance genes (e.g., on integrons [12]), but specific growth states (e.g., biofilms) and single mutations (e.g., impacting on outer-membrane permeability or the expression of broadly specific multidrug efflux systems) can also promote multiresistance [13]. This review highlights recent advances in our understanding of efflux-mediated multiresistance in Gram-negative bacteria, including updates on the distribution of efflux systems in these organisms, their substrate profiles, and their contribution to antimicrobial resistance in clinical

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strains. The ongoing discussions concerning the natural function of these efflux systems and their possible involvement in biocide-mediated selection of antibiotic-resistant strains will also be highlighted. The interested reader is referred to other recent reviews for a more comprehensive treatment of the subject [13,14].

MULTIDRUG EFFLUX

While efflux as a mechanism of resistance to individual agents has been known for some time, multidrug efflux systems have only recently been identified and appreciated as significant determinants of resistance [14,15]. Chromosomally encoded and broadly distributed in Gram-negative bacteria [16], these systems play an important role in intrinsic and acquired multiresistance [13,14]. Bacterial efflux systems capable of accommodating multiple antimicrobials fall into five classes: the major facilitator superfamily (MFS); the ATP-binding cassette (ABC) family; the resistance-nodulation-division (RND) family; the small multidrug resistance (SMR) family (a member of the much larger drug/metabolite transporter (DMT) superfamily); and the multidrug and toxic compound extrusion (MATE) family [17]. Although not unique to Gram-negative bacteria, RND family transporters are most commonly found in such organisms and typically operate as part of a tripartite system that includes a periplasmic membrane fusion protein (MFP) and an outer-membrane protein (now called outer-membrane factor (OMF)) [18], an organisation also seen on occasion with MFS (e.g., EmrAB-TolC; see below) and ABC (e.g., the macolide-specific MacAB-TolC efflux system [19]) transporters (Fig. 1). Members of all but the ABC family (whose members hydrolyse ATP to drive drug efflux) function as secondary transporters, catalysing drug–ion (H^+ or Na^+) antiport (Fig. 1).

RND FAMILY OF MULTIDRUG EFFLUX SYSTEMS

The most relevant multidrug efflux systems in terms of resistance to clinically important agents (including antibiotics and biocides) are members of the RND family [13] (Table 1), although members of the MFS, MATE and SMR families also show a limited ability to promote resistance to some biocides and antibiotics (Table 2). Originally identified as determinants of fluoroquinolone resistance in a number of Gram-negative pathogens [20], efflux systems of the RND–MFP–OMF type are known to accommodate a broad range of structurally unrelated molecules that can include most classes of antibiotics, as well as biocides, dyes, detergents, metabolic inhibitors, aromatic hydrocarbons (i.e., organic solvents), cationic antimicrobial peptides, toxic fatty acids, bile salts, and homoserine lactones associated with quorum-sensing [13,21]. These last play a role in cell density-dependent expression of a number of virulence factors in (for example) *P. aeruginosa*, and thus the activity of these efflux systems can influence virulence [22,23]. Indeed, a recent study suggests that the MexAB–OprM efflux system of *P. aeruginosa* may promote the release of molecule(s) important for the virulence of this organism [24].

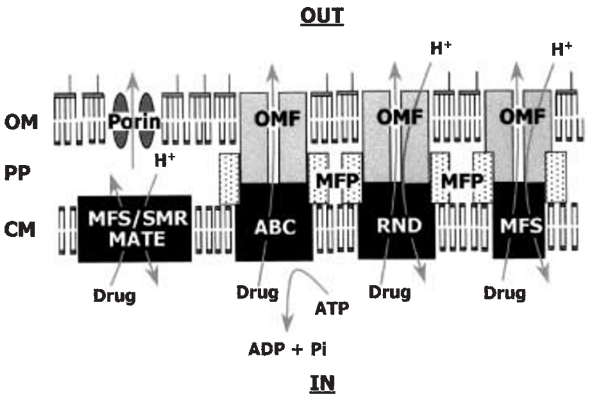

Fig. 1. The organisation and operation of antimicrobial efflux pumps of Gram-negative bacteria. OM, outer membrane; PP, periplasmic space; CM, cytoplasmic membrane.

RND–MFP–OMF multidrug efflux systems contributing to antimicrobial resistance have been described in a broad range of Gram-negative pathogens (human, animal, plant) and non-pathogens (Table 1). In view of the role played by RND–MFP–OMF-type multidrug efflux systems in efflux-mediated fluoroquinolone resistance, reports of efflux-mediated resistance to fluoroquinolones in *Vibrio cholerae* [25], *Citrobacter freundii* [26] and *Proteus vulgaris* [27] may also be indicative of multidrug efflux systems in these organisms. The best characterised efflux systems of the RND–MFP–OMF group are those found in *Escherichia coli* [28], *P. aeruginosa* [21] and *Neisseria gonorrhoeae* [29], and homologues of these systems are readily identifiable in both finished genome sequences (see Table 1 for

Table 1. RND family multidrug efflux systems of Gram-negative bacteria

Organism	Efflux components[a]			Regulatory genes	Expression[b]	Substrates[c]	Reference(s)
	MFP	RND	OMF				
Acinetobacter baumannii	AdeA	AdeB	AdeC	*adeST*[d]	wt/+	AG, CX, TC, ER, CM, TP, FQ	[45]
Agrobacterium tumefaciens	AmeA	AmeB	AmeC	*ameR*	wt/+	BS, NV, SDS	[102]
Brucella suis	AcrA[e] (AAN34087)	AcrB[e] (AAN34086)	?	*gntR*[f] (AAN34085)	?	?	
Brucella melitensis	AcrA[e] (AAL52073)	AcrB[e] (AAL52074)		*acrR*[f] (AAL52072)	?	?	
Burkholderia cepacia	CeoA (U97042)[g]	CeoB (U97042)[g]	OpcM	?	wt/–; mutant/+	CM, CP, TP	–[h]
Burkholderia pseudomallei	AmrA	AmrB	OprA	*amrR*	wt/+	ML, AG	–[h]
Campylobacter jejuni	CmeA	CmeB	CmeC	?	wt/+; mutant/?	FQ, ER, BL, RF, TC, EB, SDS, DOC, CM, GN, ACR	[103,104]
Caulobacter crescentus	AcrA[e] (AAK22793)	AcrB[e] (AAK22792)	?	?	?	?	
Enterobacter aerogenes	AcrA	AcrB	TolC	*acrR*	wt/?; mutant/++	FQ, CM, TC, ACR, NOV, SDS, BS	[32]
Enterobacter cloacae	?	AcrB	?	?		CIP	[33]
Escherichia coli K12	AcrA	AcrB	TolC	*acrR*, *marA*, *robA*, *soxS*, *sdiA*[l]	wt/+; *marR*/++[j]; *acrR*/++	AH[k], BA, BL, NV, ER, FU, FQ, TC, CM, EB, AC, CV, SDS, TX, BS, TS, PO, FA, MX[k], LN[k]	–[h]
	AcrE	AcrF	TolC	*acrS*, *sdiA*[l]	wt/–; mutant/+	AH, FQ, TC, TP, LN, ML, BS, SDS, AC, RD, BL[m]	–[h]
	AcrA	AcrD	TolC	*sdiA*[l], *baeR*[kk], *cpxR*[k], *ompR*[kk]	wt/+	AG, NV, DOC, SDS[n] BL[ll]	–[h]
	MdtA (YegM)	MdtB/MdtC (YeN/YegO)	TolC	*baeSR*	wt/–[o]	NV, BL[ll], BS, SDS°	[54,105,106]
	YhiU	YhiV	TolC	*evgA*	wt/–[o]	ER, NV, DOX, CV, EB, SDS, BS, BA, RD[o], BL[ll]	[54,107–109]
Escherichia coli O157:H7	AcrA[p] (BAB33939)	AcrB[p] (BAB33938)	TolC[p] (BAB37346)	*acrR*[p] (BAB33940)	?	?	
	?	AcrD[p] (BAB36755)	?	?	?	?	
Haemophilus influenzae	AcrA	AcrB	?	?	wt/+	RF, ER, NV, EB, AC, CV, SDS[q]	–[h]
Klebsiella oxytoca	AcrA	AcrB	?	?	wt/?; mutant/+	FQ	[34]
Klebsiella pneumoniae	AcrA[p] (AJ318073)	AcrB[p] (AJ318073)	?	*acrR*[p] (AJ318073)	wt/?; mutant/+	FQ	[34]
Neisseria gonorrhoeae	MtrC	MtrD	MtrE	*mtrR*, *mtrA*	wt/+; *mtrR*/++ *mtrA*/–	AZ, ML, RF, PN, TX[r], CV, CP	–[h]

Table 1. continued

Organism	Efflux components[a]			Regulatory genes	Expression[b]	Substrates[c]	Reference(s)
	MFP	RND	OMF				
Neisseria meningitidis	MtrC (CAB85190)	MtrD (CAB85189)	MtrE (CAB85188)	*mtrR* (CAB85191), *mtrA* (CAB85011)	?	?	
Porphyromonas gingivalis	XepA	XepB	XepC	?	wt/+	RF, PM, EB	[110]
Proteus mirabilis	AcrA	AcrB	?	*acrR*	wt/+	TG, MN, CP, NV, CM, ER, EB, AC, TP, SDS	[80]
Pseudomonas aeruginosa	MexA	MexB	OprM	*mexR*	wt/+; *nalB*/+++; *nalC*[s]/++;	BL[t], FQ, CM, NV, TP, TG[t] SM, EB, AC, CV, SDS, AH, HL, CL, TL, IR, TS, TPP, RD	–[h]
	MexC	MexD	OprJ	*nfxB*	wt/–[u]; *nfxB*/++	BL[v], FQ, CM, NV, TP, TG[t], SM, EB, AC, SDS, AH, CL, TS, TG[v], TPP, RD	–[h]
	MexE	MexF	OprN	*mexT*	wt/–; *nfxC*[w]/++	FQ, CM, TP, AH, TS	–[h]
	MexX (AmrA)	MexY (AmrB)	OprM	*mexZ* (*amrR*)	wt/+	BL[x], FQ, AG, TC, ER, TG[x]	–[h]
	MexJ	MexK	OprM	*mexL*	wt/–; mutant/+	TS[y], ER, TC, CP	[57]
	MexH	MexI	OpmD	?	wt/?; mutant/+	VA, HL?, EB, NOR, RD, AC	[111,111a]
	MexV	MexW	OprM	?	wt/?; mutant/+	FQ, CM, TC, ER, EB, AC	[111b]
	pa1435[z] (AAG04824)	pa1436[z] (AAG04825)	?	?	?	?	
	pa3523[z] (AAG06911)	pa3522[z] (AAG06910)	pa3521[z] (AAG06909)	?	?	?	
	pa0156/ pa0157[z] (AAG03546/ AAG03547)	pa0158[z] (AAG03548)	?	?	?	?	
	pa2528[z] (AAG05916)	pa2526/ pa2527[z] (AAG05914/ AAG05915)	pa2525[z] (AAG05913)	?	?	?	
Pseudomonas putida	ArpA	ArpB	ArpC	*arpR*	wt/+	TC, CM, CB, ST, ER, NV	–[h]
	MepA	MepB	MepC	*mepR*	?	BL, TC, NV, ER, AH	–[h]
	SrpA	SrpB	SrpC	*srpR*, *srpS*	wt/+[aa]	AH	–[h]
	TtgA	TtgB	TtgC	*ttgR*[ii]	wt/+	CM, AP, TC, TO	–[h]
	TtgD	TtgE	TtgF	?	wt/+[aa]	AH	–[h]
	TtgG	TtgH	TtgI	*ttgV*[jj]	wt/+; inducible ++[bb]	AH, AP, CB	–[h]

Table 1. continued

Organism	Efflux components[a]			Regulatory genes	Expression[b]	Substrates[c]	Reference(s)
	MFP	RND	OMF				
Salmonella enterica serovar Typhimurium	AcrA[P] (AAL19430)	AcrB[P] (AAL19429)	TolC[P] (AAL22060)	*acrR*[P] (AAL19431)	wt/+; mutant/++	BS, SDS, DOC, CH, TX, CV,	–[h]
	?	AcrD[P] (AAL21375)	?	*soxRS*[cc]	*soxR*/++	AC, FA, NV, ER, RF, TC, CM, NOR, NAL, BL, FQ[dd]	
Salmonella enterica serovar Typhi	AcrA[P] (CAD04961)	AcrB[P] (CAD04960)	TolC[P] (CAD07712)	*acrR*[P] (CAD04962)	?		
	?	AcrD[P] (CAD07711)	?	?	?		
Serratia marcescens	AcrA	AcrB	?	?	?	FQ	[47,112]
	SdeX	SdeY	?	?	?	ER, TC, NOR, BA, EB, AC, RD	[112a]
Shewanella oneidensis	AcrA[e] (AAN57652)	AcrB[e] (AAN57651)	TolC	?	?	AQDS[ee]	
Shigella flexneri	AcrA[P] (AAN42063)	AcrB[P] (AAN42062)	TolC[P] (AAN44553)	*acrR*[P] (AAN42064)	?		
Stenotrophomonas maltophilia	SmeA	SmeB	SmeC	*smeRS*	?	BL, AG, FQ	–[h]
	SmeD	SmeE	SmeF	*smeT*[ff]	wt/+[gg]; mutant/++	TC, ER, FQ,	–[h]
Vibrio cholerae	?	?	TolC[hh]	?	wt/+	EB, ER, NV, TX	–[h]
	AcrA[e] (AAF96540)	AcrB[e] (AAF96539)	?	?	?	?	
Xanthamonsa campestris	AcrA[e] (AAM41645)	AcrB[e] (AAM41646)		*acrR/tetR*[f] (AAM41644)	?	?	
	MexA[e] (AAM41955)	MexB[e] (AAM41954)	OprM[e] (AAM41953)	*acrR/tetR*[f] (AAM41956)	?	?	
Xylella fastidiosa	AcrA[e] (AAF85183)	AcrB[e] (AAF85184)	?	?	?	?	
	AcrA[e] (AAF83052)	AcrB[e] (AAF83056)	?	?	?	?	
Yersinia pestis	AcrA[P] (CAC92367; AAM84631)	AcrB[P] (CAC92368; AAM84630)	TolC[P] (CAC89517)	*acrR*[P] (CAC92366; AAM84632)	?	?	
	AcrA[e] (AAM84290)	AcrB[e] (AAM84291)	?	?	?	?	
		AcrD[P] (CAC92285)	?	?	?	?	

[a]Where the efflux genes/gene products have not been published or their role in antimicrobial efflux and resistance has not been verified, the GenBank protein (three-letter prefix) or gene (one-letter prefix) accession number is shown in parentheses.
[b]wt/+, efflux system is known to be expressed in wild-type cells (under laboratory growth conditions); wt/+ mutant/++, efflux system is expressed in wild-type cells but expression is enhanced in resistant strains; wt/− mutant/+, efflux system is not expressed in wild-type cells but is expressed in resistant strains. In instances where the nature of the mutation leading to enhanced efflux gene expression is known, the gene is indicated along with the relative level of gene expression.
[c]AC, acriflavine; ACR, acridines; AG, aminoglycosides; AH, aromatic hydrocarbons; AP, ampicillin; AQDS, anthaquinone-2,6-disulphonate; AZ, azithromycin; BA, benzalkonium; BL, β-lactams; BS, bile salts; CB, carbenicillin; CH, cholate; CL, cerulenin; CM, chloramphenicol; CP, ciprofloxacin; CV, crystal violet; CX, cefotaxime; DOC, deoxycholate; DOX, doxyrubicin; EB, ethidium bromide; ER, erythromycin; FA, fatty acids; FU, fusaric acid; FQ, fluoroquinolones; GN, gentamicin; HL, homoserine lactones; IR, irgasan; LN, linezolid; ML, macrolides; MN, minocycline; MX, methotrexate; NAL, nalidixic acid; NOR, norfloxacin; NV, novobiocin; PM, puromycin; PN, penicillin; PO, pine oil; RD, rhodamine; RF, rifampicin; SDS, sodium dodecyl sulphate; SM, sulphonamides; ST, streptomycin; TC, tetracycline; TG, tigecycline; TL, thiolactomycin; TO, toluene; TS, triclosan; TP, trimethoprim; TPP, tetraphenyl phosphonium; TX, Triton X-100; VA, vanadium. In instances where only one member of a class of antimicrobial has been tested or is known to be a substrate for a given pump, that member is identified. Where several members of an antimicrobial class are known to be substrates, the class is identified rather than the actual compounds tested.

Table 1. footnotes continued

[d]The *adeST* genes, encoding homologues of the sensor kinase/response regulator members of the superfamily of bacterial two-component regulators, were identified upstream of the *ameABC* efflux genes and implicated in regulating efflux gene expression. No experimental evidence supporting this was provided.
[e]Homologues of the *E. coli* AcrAB efflux components were identified by a tBlastn search of the genome sequences of the indicated organisms and the corresponding GenBank protein accession number shown in parentheses.
[f]Examination of gene sequences flanking the corresponding *acrAB*-like efflux genes in the genome sequences of the indicated organisms identified homologues of regulatory genes of the indicated family upstream of the efflux genes, implicating them in the regulation of these efflux genes.
[g]The *ceoAB* genes (accession number provided in parentheses) and their contribution to efflux-mediated multiresistance genes have not yet been published.
[h]Except where indicated, accession numbers and references in support of efflux/regulatory gene identification and substrate profiles are provided in recent review articles [13,14,20,21].
[i]*sdiA* encodes a quorum-sensing regulator that positively regulates efflux gene expression [113,114].
[j]MarR negatively regulates *marA* gene expression, such that mutations in *marR* (in so-called *mar* mutants) lead to increased MarA production and, thus, increased *acrAB* expression.
[k]Contribution of AcrAB–TolC to resistance to aromatic hydrocarbons [115], methotrexate [116] and linezolid [117,117a] has recently been confirmed.
[l]*sdiA* encodes a quorum-sensing regulator that positively influences efflux gene expression.
[m]Contribution of AcrEF–TolC to resistance to antibiotics [117], dyes and detergents [54] and aromatic hydrocarbons [118] has recently been demonstrated.
[n]Contribution of AcrD to resistance to non-aminoglycosides has recently been reported [54].
[o]The efflux genes are not expressed and do not contribute to antimicrobial resistance in wild-type cells, although their cloning on a multicopy plasmid promotes resistance to (and probably efflux of) the indicated agents.
[p]Orthologues of the *acrAB* efflux genes were identified by a tBlastn search of the genome sequences of the indicated organisms and the corresponding GenBank protein accession number shown in parentheses.
[q]Data concerning substrate profile were ascertained by examining the susceptibility of an *acrAB* deletion mutant. It is not clear, however, if the apparent differences in substrate profiles between AcrAB in *H. influenzae* and its homologues in *E. coli* and *S. enterica* are real, or whether this pump actually accommodates many more substrates but its highly permeable outer membrane compromises measurable net efflux in this organism.
[r]Full MtrCDE-mediated resistance to Triton X-100 requires the product of the recently identified *mtrF* gene, encoding a probable cytoplasmic membrane protein of 56 kDa containing 12 transmembrane domains [119].
[s]The *nalC* gene (also known as pa372) as designated by the *Pseudomonas* Genome Project (http://www.pseudomonas.com) is unlinked to the *mexAB–oprM* genes, but encodes a TetR family repressor whose inactivation leads to *mexAB–oprM* hyperexpression.
[t]MexAB–OprM accommodates many penicillins and cephems [120], provides modest resistance to a few carbapenems (not imipenem) [121] and is the major efflux system contributing to penem resistance in *P. aeruginosa* [122]. Recently, MexAB–OprM-mediated resistance to the glycylcycline tigecycline has been reported [78].
[u]Although MexCD–OprJ is not expressed in wild-type cells under normal growth conditions, it is inducible by several of its known substrates, including ethidium bromide, acriflavine, rhodamine 6G, tetraphenylphosphonium chloride, benzalkonium chloride and chlorhexidine [84,84a].
[v]MexCD–OprJ accommodates many penicillins and cephems [120], provides modest resistance to a limited number of carbapenems (not imipenem) [121], and promotes modest resistance to penems in the absence of MexAB–OprM [122]. A contribution to tigecycline resistance has also now been reported [78].
[w]The *nfxC* gene has not yet been identified.
[x]MexXY–OprM accommodates several penicillins and cephems [120] and provides modest resistance to a limited number of carbapenems (not imipenem) [121].
[y]Efflux of triclosan, but not the other antimicrobials, is provided by MexJK alone without OprM involvement.
[z]Homologues of the known *P. aeruginosa* multidrug efflux systems were identified by a blast search of the *P. aeruginosa* genome sequence and are identified by the 'pa' designations provided by the *Pseudomonas* Genome Project (http://www.pseudomonas.com) and the GenBank protein accession numbers (in parentheses).
[aa]Expression is inducible by aromatic hydrocarbons.
[bb]Expressed in wild-type cells with enhanced expression occurring in the presence of aromatic hydrocarbons.
[cc]See Koutsolioutsou *et al.* [123].
[dd]Contribution of AcrAB–TolC to fluoroquinolone resistance has recently been reported [124].
[ee]TolC-dependent export of AQDS (anthaquinone-2,6-disulphonate) has been demonstrated [125], although it is not clear whether by a mechanism that involves the AcrAB homologues identified in *Shewanella oneidensis*.
[ff]*smeT* encodes a repressor of *smeDEF* expression [126].
[gg]SmeDEF contributes to intrinsic multiresistance in *S. maltophilia* [127].
[hh]While TolC has been implicated in resistance to the indicated agents in *V. cholerae*, it is unclear if the AcrAB homologues identified in this organism work with TolC in providing this resistance.
[ii]Mediates drug inducibility of TTgABC [127a].
[jj]Encodes a repressor of *ttgGHI* expression [127b].
[kk]See [127c]. [ll]see [127d].

Table 2. Non-RND family multidrug efflux systems of Gram-negative bacteria

Source	Efflux components				Regulatory gene(s)	Expression[a]	Substrates[b]	Reference(s)
	ABC	MATE	MFS	SMR				
Chromosomal								
Bacteroides fragilis	–	–	NorA?	–	?	wt/+; mutant/++	NOR, EB, PM	–[c]
Bacteroides thetaiotaomicron	BexA	–	–	–	?	wt/+; mutant?	FQ, EB	[128]
Bordetella pertussis	–	–	–	BPsmr	?	?	MV, TPP, EB, ACR	[129]
Brucella melitensis	–	NorMI	–	–	?	?	FQ, GN, TPP, ACR, BER	[130]
Burkholderia cepacia	–	–	BcrA	–	?	?	TC, NAL	[131]
Burkholderia vietnamiensis	–	NorM	–	–	?	?	NOR	[132]
Escherichia coli	–	–	EmrB[d]	–	*emrR* (*mprA*)	wt/–; mutant/?[e]	NAL, PMA, HU, TL	–[c]
	–	–	EmrD	–	?	wt/–; mutant/?[e]	HU	–[c]
	–	–	–	EmrE (MvrC)	?	wt/+	EB, ACR, MPP, MV, TC, TPP	–[c]
	–	–	MdfA (CmlA and Cmr)	–	?	wt/+	AG, BA, CM, DM, EB, ER, FQ, IPTG, PM, RD, RF, TC, TPP	–[c]
	–	–	–	TehA[f]	?	wt/–; mutant/?[e]	CV, EB, PF, TPC	–[c]
	–	YdhE (NorM)	–	–	?	?	NOR, CP, AC, TPP, AG	–[c]
Neisseria gonorrhoeae		NorM			?	?	EB, AC, NOR, CP, BA	[133]
Neisseria meningitidis	–	NorM	–	–	?	?	EB, AC, NOR, CP, BA	[133]
Pseudomonas aeruginosa	–	–	–	EmrE	?	wt/+	EB, ACR, MV, AG	[129,134]
Vibrio cholerae	–	–	VceB[g]	–	?	wt/+	DOC, CCCP, CM, ER, PMA, PCP, NAL	–[c]
Vibrio parahaemolyticus	–	NorM[h]	–	–	?	?	NOR, CP, AG, EB	–[c]
		VmrA			?	?	TPP, ACR, EB	[135]
Plasmid-encoded								
	–	–	–	QacE	?	NA[j]	EB, PF, CV, QAC	–[c]
	–	–	–	QacEΔ1	?	NA[j]	EB, PF, CV, QAC	–[c]
	McbF[i]	–	–	–	*emrR* (*mprA*)	NA[j]	FQ, HU	–[c]

[a]Relative expression of the corresponding efflux system is indicted in wild-type (wt) cells (where known) and in mutants known to express the system. In instances where the nature of the mutation leading to enhanced efflux gene expression is known, the gene is indicated along with the relative level of gene expression. wt/+ mutant/++, efflux system is expressed in wild-type cells (under laboratory growth conditions) but expression is enhanced in resistant strains; wt/– mutant/+, efflux system is not expressed in wild-type cells but is expressed in resistant strains; ?, expression status unknown.

Table 2. footnotes continued

[b]AC, acriflavine; ACR, acridines; AG, aminoglycosides; BA, benzalkonium; BER, berberine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CM, chloramphenicol; CP, ciprofloxacin; CV, crystal violet; DM, daunomycin; DOC, deoxycholate; EB, ethidium bromide; ER, erythromycin; FQ, fluoroquinolones; GN, gentamicin; HU, hydrophobic uncouplers; IPTG, isopropyl-β-D-thiogalactopyranoside; MPP, methyltriphenylphosphonium; MV, methyl vialogen; NAL, nalidixic acid; NOR, norfloxacin; PCP, pentachlorophenol; PF, proflavin; PM, puromycin; PMA, phenylmercuric acetate; QAC, quaternary ammonium compounds; RD, rhodamine; RF, rifampicin; TC, tetracycline; TL, thiolactomycin; TPC, tetraphenylarsonium chloride; TPP, tetraphenyl phosphonium. In instances where only one member of a class of antimicrobial has been tested or is known to be a substrate for a given pump, that member is identified. Where several members of an antimicrobial class are known to be substrates, the class is identified rather than the actual compounds tested.
[c]Accession numbers and references in support of efflux/regulatory gene identification and substrate profiles are provided in recent review articles [13,14,20].
[d]Part of a probable three-component export system that includes the MFP component, EmrA, and the OMF component, TolC.
[e]Efflux activity and/or drug resistance has only been shown for the gene cloned on a plasmid.
[f]Part of a two-component export system that includes TehB.
[g]Part of a two-component export system that includes the MFP component VceA.
[h]First described in *V. parahaemolyticus*, NorM homologues (ascertained using BLAST searches) are found in the genome sequences of a broad range of Gram-negative organisms, including most of those listed in Table 1.
[i]Part of a two-component export system that includes McbE.
[j]Not applicable.

examples) and unfinished genome sequences (e.g., *Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella bronchiseptica*, *Burkholderia mallei*, *Legionella pneumophila*, *Pasteurella multocida* and *Pseudomonas syringae*; http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table_cgi) of a number of Gram-negative bacteria, although their contribution to antimicrobial resistance in these organisms remains to be determined. Increasingly, multidrug efflux systems of the RND–MFP–OMF type are implicated in clinical episodes involving antibiotic-resistant organisms. Examples include *E. coli* [30,31], *Enterobacter aerogenes* [32,32a], *Enterobacter cloacae* [33], *Klebsiella* spp. [34]., *N. gonorrhoeae* [35–37], *Salmonella enterica* serovar Typhimurium [38–41,41a], *P. aeruginosa* [42,43,43a,b], *S. maltophilia* [44], *Acinetobacter baumannii* [45], *Burkholderia cepacia* [46] and, possibly, *Serratia marcescens* [47] and *Campylobacter jejuni* [48]. Additional references can be found in other recent review articles [13,20,21]. Efflux-mediated fluoroquinolone resistance in clinical isolates of *C. freundii* [26], *Ent. aerogenes* [49], *Ent. cloacae* [33] and *Klebsiella pneumoniae* [50,51] may also be attributable to multidrug efflux systems.

ANTIBIOTIC–BIOCIDE CROSS-RESISTANCE

The observation that RND–MFP–OMF efflux systems in both *E. coli* (AcrAB [52–54]) and *P. aeruginosa* (MexAB–OprM [55], MexCD–OprJ [56], MexEF–OprN [56] and MexJK [57]) accommodate both biocides and clinically important antibiotics has added fuel to the debate on biocide–antibiotic co-resistance and the possible selection of multiply antibiotic-resistant strains by biocides [9,58–64,64a]. While it appears that such multidrug efflux systems are unlikely to facilitate resistance to biocides used at the recommended 'working' concentrations of these agents, the risk of residual biocide levels selecting pump-producing mutants that are concomitantly resistant to clinically relevant antibiotics is of some concern [65]. Certainly, in-vitro studies have confirmed that biocide-resistant strains which overexpress multidrug efflux systems, and thus become resistant to multiple antibiotics, can be selected in both *E. coli* [52,53] and *P. aeruginosa* [56]. Given the broad distribution of Mex- and Acr-like efflux systems in Gram-negative bacteria (see above and Table 1), the risk of efflux-mediated biocide–antibiotic cross-resistance in other pathogens is a real possibility.

EFFLUX SYNERGY WITH OTHER RESISTANCE MECHANISMS

The intrinsic and acquired resistance of Gram-negative bacteria invariably depends upon reduced antimicrobial accumulation, with or without antimicrobial modification or destruction or target site alterations [13]. The former comes about as a result of the synergy between the

outer-membrane barrier and active efflux mechanisms, and disruption of either is effective in enhancing accumulation and, thus, susceptibility [66]. The importance of this synergy is highlighted by the concomitant decline in porin production, which decreases outer-membrane permeability to, for example, hydrophilic agents, and the increase in efflux activity that is seen in so-called multiple antibiotic-resistant or *mar* mutants of *E. coli* [67]. A *mar* locus (or a Mar-like phenotype) is, in fact, widely distributed among Gram-negative bacteria [13,68,69]. Increasingly, resistance to fluoroquinolones in Gram-negative bacteria results from a combination of enhanced expression of multidrug efflux systems and target site mutations [25,39,48,70–73]. It may be that efflux was also a contributing (but overlooked) factor in earlier reports of fluoroquinolone resistance attributed to target site mutations. Significantly, loss of multidrug efflux (AcrAB in *E. coli* [74] or *Salmonella typhimurium* [39]; MexAB–OprM in *P. aeruginosa* [75]; CmeABC in *Camp. jejuni*) can undermine the resistance provided by target site mutations, in some instances rendering the mutants susceptible despite the presence of these target site mutations [39,74,75]. These results highlight both the significance of the contribution of efflux mechanisms to fluoroquinolone resistance in Gram-negative organisms, and the probable utility of efflux inhibitors in countering resistance attributable to efflux and target site mutations.

OVERCOMING EFFLUX-MEDIATED MULTIRESISTANCE

An obvious approach to overcoming resistance mediated by efflux mechanisms is to target them directly (i.e., develop efflux inhibitors) or to develop antimicrobials that are less impacted by efflux (i.e., are poor substrates for these efflux systems). To date, several inhibitors active against the multidrug efflux systems of Gram-negative bacteria have been described that are, for example, effective at reversing efflux-mediated multiresistance [4,76,77]. The glycylcycline tigecycline (GAR-936), originally identified as being unaffected by (for example) the tetracycline-specific Tet efflux determinants found in *E. coli*, also appears to be an inferior substrate for the multidrug efflux systems of *P. aeruginosa* [78] and is active against multiresistant *S. maltophilia* and *A. baumannii* [79]. Nonetheless, *P. aeruginosa* is still generally resistant to this agent, which is exported by the MexAB–OprM, MexCD–OprJ and MexXY–OprM multidrug efflux systems of this organism [78]. Moreover, the notably reduced susceptibility of *Proteus mirabilis* to tigecycline was recently explained by the activity of the AcrAB multidrug efflux system of this organism, which readily accommodates this agent [80]. The likelihood that many novel agents may be substrates for these highly accommodating efflux systems [81] needs to inform current antimicrobial strategies that focus on developing novel agents or novel drug targets [82]. Given the synergy that exists between outer-membrane impermeability and active drug efflux, targeting the outer-membrane barrier is also proposed as an effective way of compromising efflux-mediated multiresistance [13]. Finally, therapeutic approaches based on vaccines, immunomodulation, cationic antibacterial peptides and phages, to name just a few, may become increasingly important in treating multiresistant Gram-negative infections, benefiting as they do from an 'insensitivity' to drug efflux mechanisms [4].

NATURAL FUNCTION OF MULTIDRUG EFFLUX SYSTEMS

There is much discussion in the literature as to the natural function of bacterial multidrug efflux systems, with evidence for induction of the systems by agents known to be exported, providing support for proposed roles in protection against noxious exogenous substances. For example, the MexXY–OprM system of *P. aeruginosa* is both induced by and exports several antibiotics, including gentamicin, erythromycin and tetracycline [83], and, as such, efflux of these agents may be its intended function. However, it is also possible that the action of these agents on their ribosome targets stimulates the production of cellular product(s) which are the intended MexXY–OprM substrates and, thus, induce expression of this efflux. The MexCD–OprJ efflux system of this same organism is also inducible by a number of its non-antibiotic substrates (e.g., the dyes rhodamine 6G, acriflavine and ethidium bromide and the biocides benzallcon chloride and chlorhexid), although not by any clinically relevant antibiotics [84a]. The observed inducibility of the *E. coli* AcrAB system by toxic fatty acids [85], and the demonstrated role of AcrAB in the export of and

resistance to bile salts [86], have been cited as support for a role for AcrAB in protecting the cell from the action of these agents in the gut [85], although clearly there is no evidence for antibiotics being the intended substrate. Similarly, a protective function has been attributed to the MtrCDE system, which provides resistance to faecal lipids in rectal isolates of *N. gonorrhoeae* [87] and, probably, bile salts known to bathe mucous membranes [88]. Still, the ability to accommodate bile salts is not, in itself, proof of a protective function for either efflux system, inasmuch as many of the so-called multidrug efflux systems of (for example) *E. coli* accommodate and provide resistance to bile salts [54]. It would appear unlikely that a given organism would devote several systems to the export of bile salts, and so these agents may be just some of many, perhaps unintended, substrates for these highly accommodating, broadly specific efflux systems.

Export of bacterial products is also a proposed function for these multidrug efflux systems, with a previous review implicating byproducts of metabolism as probable substrates for these efflux systems [89]. The upregulation of the *E. coli* AcrAB efflux system in strains with mutations in central biosynthetic pathways, possibly as a result of accumulation of pathway intermediates [90], certainly support this idea. The fact that a homologue, IefABC, of the AcrAB and MexAB efflux systems exports isoflavanoids in the plant pathogen *Agrobacterium tumefaciens* adds support to suggestions that non-antimicrobials are the intended substrates of many of these efflux systems [91]. Finally, efflux homologues of the RND–MFP–OMF type are broadly conserved among Gram-negative organisms, with multiple examples present in many species (Table 1) that are invariably linked to regulatory genes, implying individual control of these systems and, thus, distinct functions in the cell. So, while these systems, even in a single organism, accommodate many of the same antimicrobials, it is unlikely that their export is the primary function of these systems. Still, given their contribution to resistance against many clinically relevant agents, and their broad distribution, it is clear that these efflux systems are important determinants of antimicrobial resistance in many Gram-negative pathogens, and that their operation in these organisms is likely to compromise the use of current and yet to be developed agents.

CONCLUSIONS

Multiresistance is a significant problem in medicine because it limits chemotherapeutic options. The recognition that many instances of intrinsic and acquired antimicrobial (including biocide) resistance are attributable, at least in part, to the activity of broadly specific multidrug efflux systems, and that these are broadly conserved in Gram-negative pathogens, means that antimicrobial (including biocide) use and development must be informed by the possible impact of efflux. While multidrug efflux might compromise the activity of novel agents that could be efflux substrates, a possible bigger concern is that potent new agents might be missed in, for example, whole cell screens precisely because they are excluded from the cell by these efflux mechanisms [81]. Current efforts aimed at developing multidrug efflux inhibitors [77,92] that can be used in combination with existing and, possibly, novel agents (a strategy that has proven effective in the case of β-lactam–β-lactamase inhibitors [93]) may ultimately prove important in preserving a strategy for treating infectious disease based on chemotherapeutic agents. Such efforts will be aided by recent reports of the three-dimensional structures of components of the RND–MFP–OMF pump family [94–96], and by the identification of the drug recognition regions of the RND component [97–101], both of which can assist in rational inhibitor design.

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